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13. ABSTRACT (Maximum 200 Words)

Loss of genetic material (LOH) in the chromosome 17 p13.2 at the microsatellite marker D17S796 has been identified in atypical ductal hyperplasia and in situ ductal carcinoma of the breast. Our results shown LOH at the same region in MCF-10F cells treated with the chemical carcinogen benz(a)pyrene (BP). Moreover, microcell-mediated transfer of an intact copy of human chromosome 17 inhibited the tumorigenicity of BP1E and PCR-SSCP analyzes showed a restoration of the lost material in BP1E-17-neo. These experiments suggested the presence of a tumor suppressor gene in 17p13.2 near the marker D17S796. We have been able to clone a fragment of the genes that could represent the last exon of a bigger peptide. The presence of a 3' splicing site in the putative introns and the ATTAAA region at the 3'end support this idea. The predicted amino acid sequence does not share significant homology with any known protein supporting the idea that this could be a novel protein. Further experiments will be done in order to clone the full-length cDNA and to study the regulation of the expression of this novel gene.

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A-INTRODUCTION

Breast cancer is a hormone dependent malignancy whose incidence is steadily increasing in most western societies and in countries that are becoming industrialized (1-5). In United States, breast cancer is the second to lung cancer as a cause of cancer-related deaths (1). Apoptosis (programmed cell death) is a cell suicide process that plays important roles in multiple facets of normal development and physiology (68). Deregulation of apoptosis has been correlated with degenerative diseases, autoimmune disorders and cancer. Apoptosis is caused by caspases, a family of cystein proteases that cleave target proteins at aspartyl residues (56). New studies of the biochemical mechanisms evoked by conventional treatments for neoplastic diseases point to apoptosis as a key process for elimination of unwanted cells (6). Impaired function of apoptosis-related genes is deeply involved in oncogenesis and the progression of cancers (6-10). Our laboratory has recently found a link between apoptosis in chemically transformed human breast epithelial cells and a gene/s located in chromosome 17p13.2 (13), making necessary to identify genes that may regulate apoptosis (12,14-18). For this purpose we have proposed **to isolate in the precise location in chromosome 17p13.2-p13.1 the gene (s) responsible for the control of apoptosis and to determine the functional role of the isolated gene in the process of neoplastic progression in vivo.**

B-BODY

B-i-- The experimental system.

We have developed an *in vitro* system in which the environmental carcinogen benz(a)pyrene (BP) has been utilized for inducing the transformation of human breast epithelial cells (HBEC) (19-32). For developing this paradigm of human breast cancer, we have capitalized in the availability of the mortal HBEC-MCF-10M or Sample #130, which without viral infection, cellular oncogene transfection, or exposure to carcinogens or radiation became spontaneously immortalized, originating the cell line MCF-10F (33,34). Treatment of MCF-10F cells with chemical carcinogens responded to *in vitro* treatment with BP with the expression of all the phenotypes indicative of neoplastic transformation. BP-treated MCF-10F cells expressed increased survival and formation of colonies in agar methocel, loss of ductulogenic properties in collagen matrix, invasiveness in a Matrigel *in vitro* system (clones BP-l) and tumorigenesis in severe combined immunodeficient (SCID) mice (BPI-E) (19, 22, 27).

B-ii-Background

Because chromosome 17 was involved in both the early and late stages of carcinogenesis we selected it for testing their functional roles in chemically transformed HBEC using a microcell-mediated chromosome transfer (MMCT) technique (35-38). Our study found that seven out of ten clones with chromosome 17 transferred in to BP1E cells had reverted transformed phenotypes such as advantageous in growth, colony formation in agar-methocel, loss of ductulogenesis and resistant to apoptosis (13). All together the data indicate that 17p13.2 near the marker D17S796 contains one or more gene/s controlling the transformation phenotypes. Allelic imbalance in chromosome 17p13.2 at the microsatellite marker D17S796 has been identified in hepatocellular carcinoma (54) and atypical ductal hyperplasia and in situ ductal carcinoma of breast (39, 55).

Microcell-mediated transfer of a human chromosome 17 into BP1E showed a restoration of the lost material in BP1E-17 neo. In the last progress report (Period of March 31, 2002 to April1, 2003) we suggested the presence of a gene/s that are related to the transformed phenotype in 17p13.2 near the marker D17S796 and a 940 bp of this region was amplified and cloned. Sequences analyzes has shown that cells with transformation phenotype BP1E have lost 10-12 bases consisting in a TG repetition. There is no gene already described in this region although, RT-PCR experiments shown that this region was expressed in MCF-10F, BP1E and BP1E-17 neo. Also we found that the expressed-sequence tag EST 3179739 matches a region that lays 120 bp downstream of the cloned region. The EST 3179739 sequence comes from a cDNA library from lung (tissue type: carcinoid). A 99% identity was found between both sequences using Blast (www.ncbi.nlm.nih.gov/blast/Blast.cgi). The predicted amino acid sequence does not share significant homology with any known protein supporting the idea that this could be a novel protein. In order to cloned the full-length cDNA of this gene, rapid amplification of cDNA ends (RACE) were performed. RACE is a procedure for obtaining full-length cDNA copies of low abundance mRNAs. Although, different cDNAs were obtained, none of them were specific to this region (17p13.2). Furthermore, based on these results we have pursued (i) a detailed analysis using different microsatellite markers lying near D17S796, (ii) studies on the expression of different genes near the marker D17S796 and (iii) assays to measure the functional role of them by determining the apoptotic activity of MCF-10F, BP1E and BP1E-17neo cells after been challenged by an apoptotic inducing agent. The data presented below are covering the period of April 1, 2003 to March 31, 2004:

B-iii-a- Methods and procedures.

Cell lines. The following human breast epithelial cells were used: MCF-10F (passage 126), BP1E (passage 37) and BP1E-17neo (passage 13). MCF-10F cell line is a spontaneously immortalized human breast epithelial cell line (33; 34). BP1E cell line was derived from MCF-10F transformed by the carcinogen benzo(a)pyrene (BP) (27). The BP1E cells express all the phenotypes indicative of neoplastic transformation such as colony formation in agar methocel, and loss of ductulogenesis in collagen matrix (27). BP1E was used in microcell mediated chromosome transfer by inserting the human chromosome 17 originating the BP1E-17neo. This cell line (BP1E-17neo clon II-3) was maintained in high calcium media with 5% horse serum and geneticin (400 μg/ml).

DNA isolation. DNA was prepared from MCF-10F, BP1E and BP1E-17neo (II-3). The cells were treated with lysis buffer (100 mM NaCl, 20 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS) with 200μg/ml proteinase K and incubated at 65°C for 15 minutes with gentle agitation. The samples were cooled down on ice and treated with 100 μg/ml RNase at 37°C for 30 minutes. One phenol extraction was done followed by another with chloroform: isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75M with ammonium acetate and the DNA was precipitated with 100% ethanol. The samples were centrifuged, dried and dissolved in distilled water. The DNAs were used for CGH and microsatellite analysis.

Chromosome banding and cytogenetic analysis. Cell were arrested in metaphase using colcemid at a final concentration of 0.01μg/ml and removed from the culture flask by trypsinization prior to treatment with hypotonic solution (0.075M KCl) for 20 min at 37°C. The cells were fixed in three changes of a 3:1 mixture of methanol: glacial acetic acid at –20°C. Metaphase cells were prepared by the steam-drying technique (57). Chromosomes were analyzed after G-banding. At least 20 cells were counted and five cells were karyotyped. Chromosome identification and karyotipic designations were in accordance with the ISCN (1985), as updated in ISCN (1992).

CGH Analysis. Protocols for DNA labeling and hybridization were as previously described (58; 59). Gray-level images of fluorescence were captured with a Zeiss (Thorndale, NY) microscope connected to a cooled, charge-coupled-device camera (Photometrics, Tucson, AZ). Digital image analysis was performed using the Quipps software (Vysis, Downers Grove, IL). The threshold was set at 0.8 and 1.2 for losses and gains, respectively. The mean values of individual ratio profiles were calculated from at least 7 metaphase spreads. Averaged values were plotted as profiles alongside individual chromosome ideograms. Overrepresentation exceeding a threshold value of 1.50 was designated a HLG. A HLG defined by a sharp peak was considered indicative of DNA sequence amplification.

Microsatellite analysis. The PCR reactions were carried out in a final volume of 10 μl containing 1X PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.5 pmol of each primer, 100μM dNTPs and 0.25U TaqPlatinum (Invitrogen) and 20ng DNA. The PCR products were analyzed by capillary electrophoresis using CEQ 8000 (Beckman Coulter). The forward primers were fluorescent-labeled (Proligo, CA) and the PCR conditions consisted of a denaturation step followed by 16 cycles at 94°C for 20 sec, 60°C for 45 sec (decreasing 0.5°C per cycle) and 72°C for 30 sec; 34 cycles at 94°C, 20 sec, 50°C for 45 sec and 72°C for 30 sec. The fluorescent PCR products were mixed with an internal standard size marker and fractionated using CEQ8000 (Beckman Coulter). The markers shown in Table 1 were used. Microsatellite instability (MSI) was defined as a shift of the allelic band or a change (increase or decrease) in the broadness of the allelic band and lost of heterozygosity (LOH) was defined as a total loss (complete deletion) or a 30% or more reduction in the signal of one of the heterozygous alleles compared with the control MCF-10F DNA (60).

Growth curve. The cells were plated in a 96 well plate at a density of $2x10^3$ cells in each well chamber. The quantification of cell proliferation was measured using the colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to Formazan by mitochondrial dehydrogenases (61). The cells were counted at 24, 48, 72, 96 and 120h post plating. The doubling time was calculated using a growth curve that was plotted using relative cell number as Y-axis and time as X-axis. Each experiment was performed in triplicate.

RT-PCR. Total RNAs were isolated from growing cells at 80% confluence using Trizol (Life Technologies, Inc.) according to manufacture's instructions. The RNAs were treated with DnaseI (Ambion) during 30 min at 37°C. After DnaseI inactivation, RT-PCR reactions were run using SuperScript One- Step RT-PCR with Platinum Taq kit (Invitrogen, Life Technologies) in a final volume of 50μl. Controls to check DNA contamination were also done. The reverse transcription was done at 45°C during 30 min, followed by a PCR cycle: denaturalization step at 94°C during 2min, 35 cycles of 94°C for 30 sec, 60°C for 45 sec and 72°C for 45 sec, and an extension step at 72°C during 10min. To analyze TP53 expression, the primers used amplified a 703-bp fragment between exon 5 to exon 9 (Table 2) (62). The expression of DEFCAP (death effector filament-forming Ced-4-like apoptosis protein) was analyzed using the primers indicated in Table 2 (63). These primers amplified a fragment of 322bp and 190bp corresponding to the DEFCAP-L and DEFCAP-S isoforms, respectively. To study DEFCAP expression also human total RNA from normal breast (Repository human total RNA, Cat # 15030, Ambion) and adenocarcinoma (Cat # 15031, Ambion) were used. The β-actin was used as control for equal loading of RNA and a fragment of 520bp was expected (Locus: NM_001101 bases 690 to 1200). The following conditions were used for the PCR: 1 cycle for 2 min at 94°C, 35 cycles at 94°C for 30 sec, 60°C for 45 sec and 68°C for 45 sec and 1 cycle at 72°C for 10min. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under UV light.

Real time RT-PCR. Real-time reverse- transcriptase (RT) PCR was used to quantify the initial amount of the DEFCAP mRNA in the MCF-10F, BP1E and BP1E-17neo, respectively. The RNAs were treated with DNAse I (Ambion) for 30 min at 37°C using the TaqMan methodology (64). The TATA box-binding protein (TBP, a component of the DNA-binding protein complex TFIID) was used as endogenous RNA control, and each sample was normalized on the basis of its TBP content. The Primers/Probe used were Hs00248187-m1 (DEFCAP) and Hs 00427620-m1 (TBP) from Applied Biosystems. The Abi Prism 7700 Sequence Detection System (Perkin-Elmer, Applied Biosystems) was used and the DEFCAP target message in the different samples was quantified by measuring Ct (threshold cycle). The Ct is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The comparative Ct method was used for relative quantitation of the samples Relative quantitation was performed using the comparative method (Applied Biosystems, User Bulletin #2, ABI Prism 7700 Sequence Detection System, December 11, 1997, updated 10/2001).

Apoptosis assays. The cells were washed with cold Guava Nexin Buffer and stained in a 50ul reaction volume with Guava Nexin PE and Guava Nexin 7-AAD. The stained cells were diluted to 500 μl with cold Guava Nexin Buffer and acquired on the Guava PC. Data was acquired on the using Guava PCA system using CytoSoft software. The cells were induced to undergo apoptosis by incubation with 50 μM Camptothecin for 20 hours at 37°C. One flask of cells was left untreated to provide uninduced control cells.

B-iii-b- Results.

Karyotype and CGH analysis. The cytogenetic characterization of the human breast cell lines MCF-10F, BP1E and BP1E-17neo were performed using a combination of the standard G-banding and CGH analysis (Figure 1). All the cell lines had extra genetic material on chromosome 1 at band p34 and they presented a balanced translocation between chromosome 3 and chromosome 9 t (3; 9) (p13; p21). The CGH analysis helped to identify the extra genetic material on chromosome arm 1p34 to be from 8q24.

The modal number of chromosomes of the control cell line MCF-10F was 46 and for BP1E transformed cell line was 47. BP1E had an additional isochromosome 10q (Figure 1). DNA losses were not observed in BP1E cell line using CGH. The modal chromosome number for BP1E-17neo was 48. BP1E-17neo has the same chromosomal abnormalities observed in BP1E and in addition has an extra copy of chromosomes 17 (Figure 1). It shows the same gain of chromosome 10q as seen in BP1E. The extra copy of chromosome 17, probably the one that was microinjected appears to be rearranged and it was composed of most of the p arm and a portion of 17q22-ter (Figure 1).

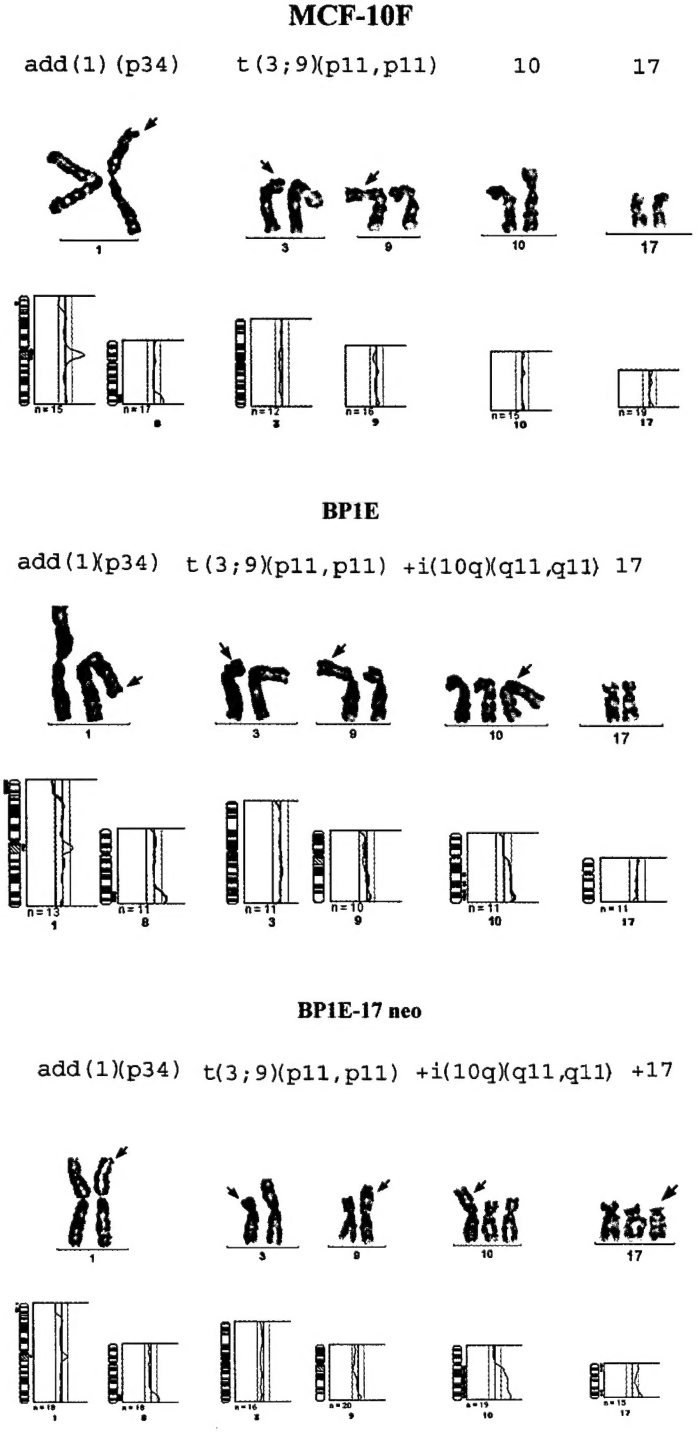


Figure 1. Karyotyping and CGH analysis of MCF-10F, BP1E and BP1E-17neo cells. *The main differences found in the G-banding and CGH analyses are included. In the three cell lines, the arrow on chromosome 1 shows the extra material at 1p34 present in the three cell lines. The arrows on chromosome 3 and chromosome 9 indicated the translocated regions between these chromosomes. The isochromosomes 10 present in BP1E and BP1E-17neo are indicated. The extra chromosome 17 present only in BP1E-17neo is also indicated. Vertical green lines on the right of each chromosome in the CGH analysis represent gains, whereas red vertical lines on the left indicate loss of genetic material.*

Microsatellite analysis. Microsatellite analysis was performed using 25 markers for chromosome 17 lying near D17S796 (Table 1). No differences were found between MCF10F, BP1E and BP1E-17neo. The PCR products obtained using the marker D17S796 were analyzed using capillary electrophoresis (Figure 2) and the only difference between the three cell lines were found with this marker located at 17p13.2 confirming our previous results (Figure 2). BP1E showed allelic imbalance in 17p13.2 using the marker D17S796, whereas, BP1E-17neo showed a pattern similar to MCF-10F. This indicated that the introduction of chromosome 17 in BP1E had reverted this mutation.

Table 1. Markers used for microsatellite studies

Marker	Primer Reverse (5′ 3′)	Primers Forward (5′ 3′)	Repeat	Location
D17S926	CCGCAGAAGGCTGTTGT	GCAGTGGGCCATCATCA	dinucleotide	17p13.3
D17S1840	TGGGGCAGACTTGGTCCTT	GCCTGGGCGACAGAGTGA	dinucleotide	17p13.3
D17S1528	CAGAGGTGGAGATAAGGG	AGTAGCCAGGAGGTCAAG	dinucleotide	17p13.3
D17S831	GCCAGACGGGACTTGAATTA	CGCCTTTCCTCATACTCCAG	dinucleotide	17p13.3
D17S1810	CCTAGTGAGGGCATGAAAC	TGTCCACTGTAACCCCTG	dinucleotide	17p13.3
D17S1832	TGTGTGACTGTTCAGCCTC	ACGCCTTGACATAGTTGC	dinucleotide	17p13.3
D17S938	ATGCTGCCTCTCCCTACTTA	GGACAGAACATGGTTAAATAGC	dinucleotide	17p13.2
D17S796	AGTCCGATAATGCCAGGATG	CAATGGAACCAAATGTGGTC	dinucleotide	17p13.2
D17S260	CTCCCCAACATGCTTTCTCT	AATGGCTCCAAAAGGAGATATTG	mononucl.	17p13.2
D17S919	GCTTAATTTTCACGAGGTTCAG	AGGCACAGAGTGAGACTTG	tetranucl.	17p13.2
D17S906	TTCTAGCAGAGTGAAACTGTCT	AGCAAGATTCTGTCAAAAGAG	tetranucl.	17p13.2
D17S1149	CGCTGATCTGTCAGGCAGCCCT	AACAAGAGTGAACTCCATAGAGAG	tetranucl.	17p13.2
D17S720	GAATTCTGAGCATATTGTTTGCCTG	CCAGCCTTGGCAACATAGCAAGA	tetranucl.	17p13.2
D17S731	TTTCTGGGGAAATTTTCTTGCTCTTA	CAACCCCAAGGTAACAACATCCAG	trinucleotide	17p13.2
D17S578	CTGGAGTTGAGACTAGCCT	CTATCAATAAGCATTGGCCT	dinucleotide	17p13.2
D17S960	TAGCGACTCTTCTGGCA	TGATGCATATACATGCGTG	dinucleotide	17p13.2
D17S1881	TAGGGCAGTCAGCCTTGTG	CCCAGTTTAAGGAGTTTGGC	dinucleotide	17p13.2
D17S1353	TACTATTCAGCCCGAGGTGC	CTGAGGCACGAGAATTGCAC	dinucleotide	17p13.2
TP53 penta	ACTCCAGCCTGGGCAATAAGAGCT	ACAAAACATCCCCTACCAAACAGC	pentanucl.	17p13.1
TP53 dint	ATCTACAGTCCCCCTTGCCG	GCAACTGACCGTGCAAGTCA	dinucleotide	17p13.1
D17S855	ACACAGACTTGTCCTACTGCC	GGATGGCCTTTTAGAAAGTGG	dinucleotide	17q21.2
D17S579	CAGTTTCATACCAAGTTCCT	AGTCCTGTAGACAAAACCTG	dinucleotide	17q21.31
D17S250	GCTGGCCATATATATATTTAAACC	GGAAGAATCAAATAGACAAT	dinucleotide	17q12
THRA1	CTGCGCTTTGCACTATTGGG	CGGGCAGCATAGCATTGCCT	dinucleotide	17q11.2
GH	TCCAGCCTCGGAGACAGAAT	AGTCCTTTCTCCAGAGCAGGT		17q22.24

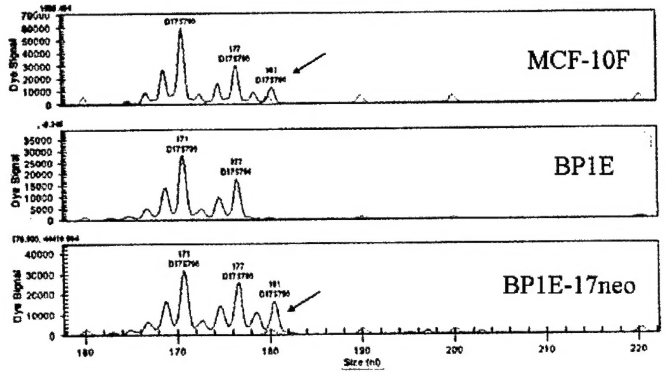
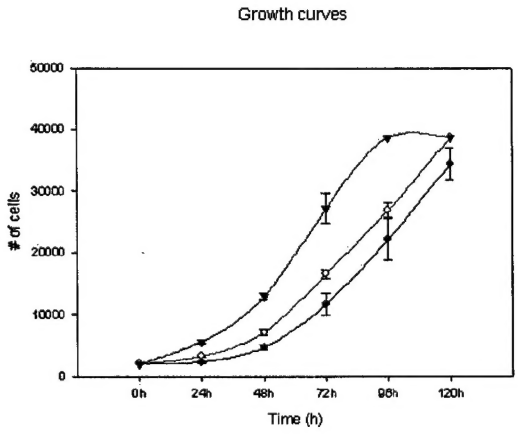


Figure 2. Microsatellite studies with D17S796 marker using capillary electrophoresis.

Growth rates. The doubling time for the different cell lines were study as another parameter of cell transformation. BP1E-17neo grew at slower rate compared with the transformed cell line BP1E (Figure 3). The doubling time for BP1E-17neo was 24h, 1.5-fold longer than the BP1E cell that has a doubling time of 16h and similar to MCF-10F that was 24.6h.



Doubling time	
BP1E	**16h**
BP1E-17neo	**24h**
MCF-10F	**24.6 h**

Figure 3. Comparative growth rates in vitro among MCF-10F, BP1E and BP1E-17neo cells. The doubling time, estimated from the growth curves, was significantly higher for BP1E-17neo (24) than for BP1E (16h). The doubling time for BP1E-17neo was similar to MCF10F (24.6h).

Analysis of the expression of DEFCAP and TP53 by RT-PCR. We have found that marker D17S796 is approximately at 1.1cM downstream of the DEFCAP gene (death effector filament-forming Ced-4-like apoptosis protein) and 1.3 cM upstream of the tumor suppressor gene TP53. DEFCAP has two isoforms, DEFCAP-L and DEFCAP-S and they differ in only 44 amino acids (63). We study TP53 and DEFCAP expression in the three cell lines by RT-PCR using the primers indicated in Table 2. We found that DEFCAP-L was downregulated in BP1E compared with MCF-10F and was overexpressed in BP1E-17neo (Figure 4). No differences were found in TP53 expression between the three cell lines (Figure 4).

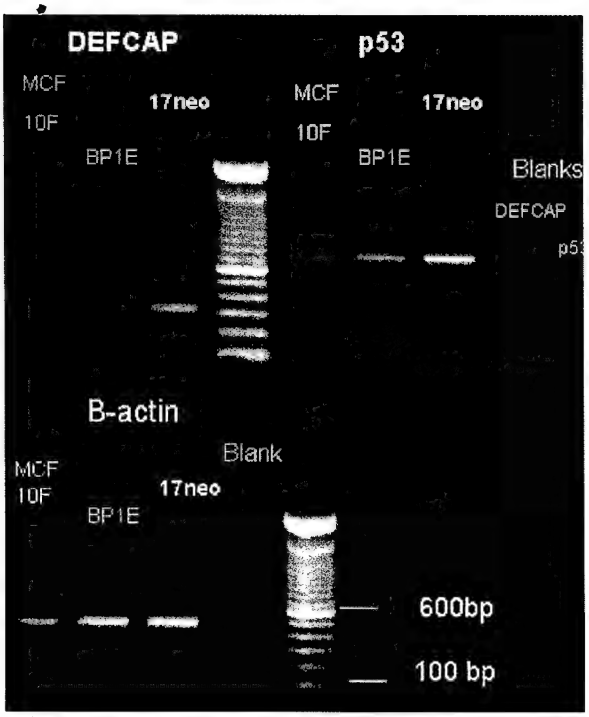


Figure 4. RT-PCR of DEFCAP and TP53 in the different cell lines. *RT-PCR using total RNA from MCF-10F, BP1E and BP1E-17 neo cells. B-actin was used as a control for equal RNA quantity used in the reactions*

Table 2. Primers used for RT-PCR

Gene	Reverse Primer (5'to 3')	Forward Primer (5' to 3')	Size (bp)
DEFCAP	TCCCCCTTGGGAGTCCTCCTGAAAATGATC	CGAGAACAGCTGGTCTTCTCCAGGGCTTCG	322 and 190
p53	TTCTTGCATTCTGGGACAGCC	GCCTCATTCAGCTCTCGGAAC	703
β-actin	GGGAAATCGTGCGTGACATTAAGG	CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC	520

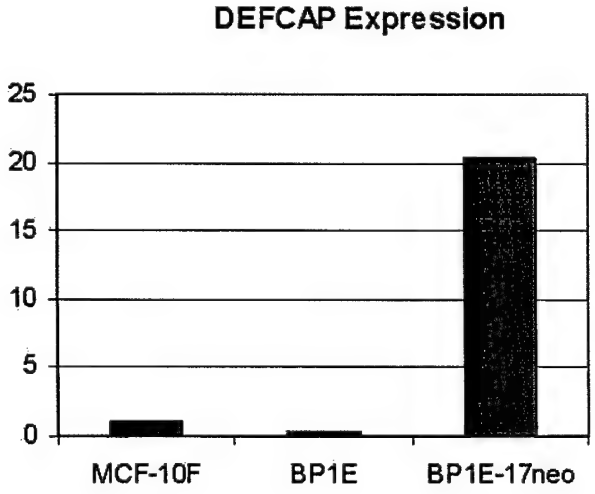


Figure 5: *Quantitation of DEFCAP expression using Real Time RT-PCR in MCF-10F, BP1E and BP1E-17 neo..*

Figure 5

Analysis of the expression of DEFCAP by Real time RT-PCR. We have used real time RT-PCR to quantify the levels of DEFCAP mRNA in MCF-10F, BP1E and BP1E-17neo cells. We found that the expression of DEFCAP in BP1E is 0.4 times less than MCF10F cells, whereas in BP1E- 17neo cells was 20 folds increased (Figure 5). Interestingly DEFCAP expression was significantly low in breast adenocarcinoma when compared with their normal counterpart (Figure 6).

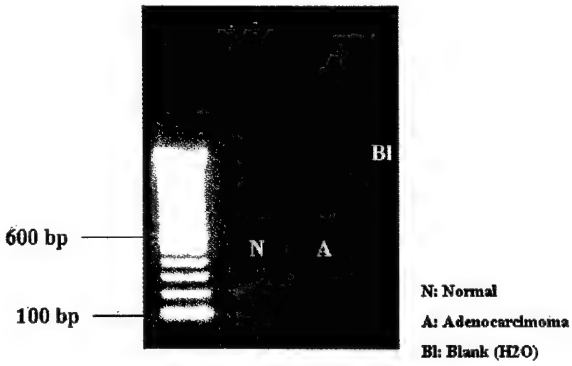


Figure 7.

Figure 6. DEFCAP expression in breast. *RT-PCR of DEFCAP in human normal breast and adenocarcinoma*

Apoptosis. The finding that DEFCAP is a gene controlling apoptosis led us to determine if the level of expression was associated with this function. For this purpose we have studied apoptosis in BP1E and BP1E-17neo using Guava Nexin procedure (Guava Technologies Inc.). This assay utilizes Annexin V – PE to detect phosphatidylserine on the external membrane of apoptotic cells. Annexin V is a calcium dependent phospolipid binding protein with a high affinity for phosphatidylserine (PS), a membrane component normally localized to the internal face of the cell membrane. Early in apoptosis, PS is translocated to the outer surface of the cell membrane where Annexin V can bind them. Apoptosis was induced using 50 μM Camptothecin and the cells were treated during 20 hs. Differences in early and late apoptosis were found between BP1E and BP1E-17 neo (Figure 7).

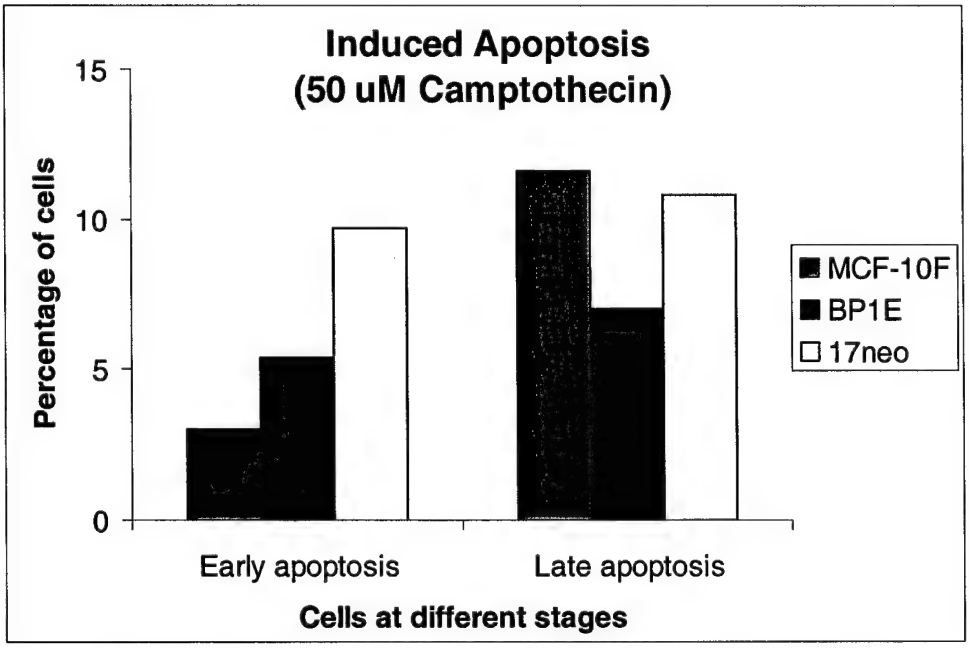


Figure 7. *Apoptosis assay. The cell lines were treated with 50μM Camptothecin for 24h to induce apoptosis. More apoptotic BP1E-17neo cells were observed in early apoptosis.*

B-iv next proposed Plan of research

We will further our understanding in the functional role of DEFCAP by treating the three cell lines under investigation with the apoptotic agent Camptothecin during 6h, 12h, 24h, 36h and 48h. Northern blots and RT-PCR experiments will be performed to study DEFCAP expression. We expect that DEFCAP expression will increase during apoptosis in MCF10F and BP1E-17neo but not in BP1E cells.

DEFCAP siRNA experiments will be performed using MCF-10F to check if the silencing of this gene affects the normal phenotype of this cell line. We expect a change in the phenotype of this cell line to a transformed phenotype similar to BP1E.

BP1E will be transfected with a plasmid harboring DEFCAP and it is expected that the over-expression of this gene in this cell will revert the transformed phenotype.

We will determine if the DEFCAP protein expression follows the same pattern in primary breast cancer than in the neoplastically transformed cells in vitro.

C-KEY RESEARCH ACCOMPLISHMENTS

- DEFCAP gene (death effector filament-forming Ced-4-like apoptosis protein) is approximately at 1.1cM downstream of the marker D17S796.
- DEFCAP also known as NALP1, NAC or CARD7, and it was the first NALP-family protein to be identified on the basis of its sequence homology to APAF-1 (63; 65; 66, 67) is down-regulated in BP1E cells and overexpressed in BP1E-neo cells in comparison with MCF10F cells.
- DEFCAP expression is associated with apoptosis and the abrogation of the neoplastic phenotype. In addition this could be relevant to the human disease since primary breast cancer has lower level of expression of this gene.
- p53 a gene close to D17S796 is not affected during the neoplastic transformation and is not modified when Ch. 17 was transferred to the transformed cells.

D-REPORTABLE OUTCOMES

1. Lareef, M.H., Fernandez, S.V., Russo, I.H., Balsara, B.B., Testa, J. and Russo, J. Role of 17p13.2 in the neoplastic transformation of human breast epithelial; cells. Proc. Am. Assoc. Cancer Res.2004.
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E-CONCLUSIONS

All together the data indicate that 17p13.2 near the marker D17S796 contains the DEFCAP gene that when inactivated is associated with the expression of cell transformation phenotypes, and that *in vitro* condition are expressed as increase doubling time, colony formation in semisolid media, loss of the ability to from ductules in collagen matrix, loss of the response to apoptosis inducing agent, and *in vivo* has been associated with ductal hyperplasia and carcinoma in situ of the breast (39) that are early stages of breast cancer.

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Appendices

CLONING OF A NEW GENE/S IN CHROMOSOME 17P13.2-13.1 THAT CONTROL APOPTOSIS
JOSE RUSSO, M.D., F.C.A.P., FASCP

Publications:

1-Lareef, M.H., Fernandez, S.V., Russo, I.H., Balsara, B.B., Testa, J. and Russo, J. Role of 17p13.2 in the neoplastic transformation of human breast epithelial; cells. Proc. Am. Assoc. Cancer Res.2004.

2.-Fernandez, S.V., Lareef, M.H., Russo, I.H., Balsara, B.B., Testa, J. and Russo, J. Estrogen and its metabolite 4-OH-E2 induce LOH at 13q12.3, at a locus 0.8cM from the BRCA2 gene in human breast epithelial cells. Proc. Am. Assoc. Cancer Res.2004.

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#4274 **Role of 17p13.2 in the neoplastic transformation of human breast epithelial cells.** Mohamed Lareef, Sandra V. Fernandez, Irma Russo, Binaifer R. Balsara, Joseph Testa, Jose Russo. *Fox Chase Cancer Center, Philadelphia, PA.*

Genomic alterations on chromosome 17 play an important role in breast cancer development. To further investigate the role of chromosome 17 in the initiation and progression of breast cancer, we have used an *in vitro* experimental system in which a human chromosome 17 was introduced into BP1E, a transformed cell line derived from benzo(a)pyrene (BP)-treated human breast epithelial cells MCF-10F (Carcinogenesis 14:483-492,1993). Microcell mediated chromosome transfer technique (MMCT) was used for chromosome transfer. MCF-10F cells do not form colonies in agar methocel and form ductules in collagen, whereas the transformed BP1E cells do form colonies and have lost their ductulogenic capacity. Transfer of chromosome 17 originated BP1E-17neo cells, which, like MCF-10F cells, did not form colonies in agar methocel, formed ductules in collagen, and their doubling time was reduced two-fold below the values observed in BP1E cells. Cytogenetic characterization of the cell lines MCF-10F, BP1E and BP1E-17neo was performed using a combination of the standard G-banding analysis and comparative genomic hybridization (CGH). BP1E-17neo cells exhibit the same chromosomal abnormalities observed in BP1E cells, having an additional chromosome 17. The extra copy of chromosome 17 appeared to be rearranged, containing most of the p arm and a portion of 17q. CGH analyses showed that it had only the most telomeric region of the q arm (q24.3 through q25.2). Microsatellite analysis performed using more than 30 markers for chromosome 17 revealed that the transformed cell line BP1E had an allelic imbalance on chromosome 17 p13.2 (D17S796), and that the transfer of chromosome 17 abrogated the transformed phenotype and corrected the allelic imbalance on this locus. Allelic imbalances in chromosome 17p13.2 (D17S796) have been identified by others investigators in atypical ductal hyperplasia and ductal carcinoma *in situ* of the breast (J. Pathol. 187:272-278, 1995). In summary, transfer of chromosome 17 suppressed the growth and transformation phenotypes of BP1E cells indicating that chromosome 17 p13.2 (D17S796) hosts one or more tumor suppressor genes that could be involved in early stages of breast cancer. (This work was supported by DAM17-01-0249 and DAM17-02-1-0384).

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#7 **Estrogen and its metabolite 4-OHE$_2$ induce LOH at 13q12.3, at a locus 0.8cM from the *BRCA2* gene, in human breast epithelial cell lines.** Sandra V. Fernandez, Mohamed Lareef, Irma H. Russo, Binaifer R. Balsara, Joseph R. Testa, Jose Russo. *Fox Chase Cancer Center, Philadelphia, PA.*

An elevated incidence of breast cancer in women has been associated with prolonged exposure to high levels of estrogens. Our laboratory has demonstrated that treatment of the immortalized human breast epithelial cells MCF-10F with 17β-estradiol (E_2) or its metabolites 4-hydroxyestradiol (4-OHE$_2$) and 2-hydroxyestradiol (2-OHE$_2$) induces phenotypical changes indicative of neoplastic transformation. E_2, 4-OHE$_2$ and 2-OHE$_2$ treated MCF-10F cells form colonies in agar methocel and lose their ductulogenic capacity in collagen matrix, expressing phenotypes similar to those induced by the carcinogen benz(a)pyrene. For investigating whether the expression of estrogen-induced transformation was associated with genomic changes, MCF-10F cells were treated with either E_2, 2-OHE$_2$, or 4-OHE$_2$ at the following doses: 0.007nM, 70 nM, or 3.6μM each. DNA was extracted and analyzed by Comparative Genomic Hybridization (CGH), which revealed that only cells treated with 4-OHE$_2$ at the 3.6μM dose exhibited gain in copy number in small regions of chromosomes 9q34, 17p12-13, 17q25, 20q13, and 19p, and sporadic losses at 13q21. No aneuploidy was observed in the treated cells. Since microsatellite instability (MSI) and loss of heterozygosity (LOH) in chromosome 13 have been reported in human breast carcinomas, we tested these parameters in MCF-10F cells treated with E_2, 2-OHE$_2$, or 4-OHE$_2$ alone or in combination with the antiestrogen ICI182-780 (ICI) at the same doses shown above. PCR reactions were analyzed by capillary electrophoresis with the CEQ 8000 (Beckman Coulter) using more than 10 microsatellite markers for chromosome 13. MCF-10F cells treated with all doses of E_2 and 4-OHE$_2$ either alone or in combination with the antiestrogen ICI, exhibited LOH in the region 13q12.3 when using the marker D13S893, which is at approximately 0.8cM upstream of the heritable breast cancer susceptibility gene BRCA2, with complete loss of one of the alleles in this region. Our results indicate that estrogen and its metabolites are mutagenic in breast epithelial cells, a phenomenon that is independent of the estrogen receptor pathway. (This work was supported by grants DMAD17-00-1-0247, DMAD17-03-1-0229).